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FABRICATION OF SINGLE CELL PROTEIN FROM CELLULOSIC WASTES

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Louisiana State University

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Techniques to produce a protein isolate from a cellulolytic bacteria were researched with the intent of producing a potential human food supplement. The use of chemical additives which cause the cells to flocculate enabling them to be removed from the fermentation media by a desludging centrifuge offers an inexpensive means of cell harvesting. Several flocculating agents which are effective at a concentration of 0.1% for flocculating Cellulomonas are reported. The addition of food grade proteins followed by a pH adjustment offers an alternative to chemical flocculation. In addition, acid and alkaline hydrolysis to remove nucleic acids from the bacterial protein was investigated.

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FOREWORD

Man and his environment must be protected from the adverse effects of pesticides, radiation, noise and other forms of pollution, and the unwise management of solid waste. Efforts to protect the environment require a focus that recognizes the interplay between the components of our physical environment--air, water, and land. The National Environmental Research Centers provide this multidisciplinary focus through programs engaged in

- studies on the effects of environmental contaminants on man and the biosphere, and
- a search for ways to prevent contamination and to recycle valuable resources.

In an attempt to solve many of the problems associated with solid waste, the synthetic foods study group at Louisiana State University has been actively engaged in developing a microbial process for producing single cell protein from cellulosic waste. The application of single cell protein as food supplements has been demonstrated and many of the problems encountered in the production of unconventional protein foods investigated.

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ABSTRACT

Techniques to produce a protein isolate from a cellulolytic bacteria were researched with the intent of producing a potential human food supplement.

The use of chemical additives which cause the cells to flocculate enabling them to be removed from the fermentation media by a desludging centrifuge offers an inexpensive means of cell harvesting. Several flocculating agents which are effective at a concentration of 0.1% for flocculating Cellulomonas are reported. The addition of food grade proteins followed by a pH adjustment offers an alternative to chemical flocculation.

Acid and alkaline hydrolysis to remove nucleic acids from the bacterial protein was investigated. Acid hydrolysis reduces the nucleic acid content to less than 2%. Alkaline hydrolysis can be combined with a spinning process to simultaneously remove nucleic acids while forming texturized protein. A heat-shocking process for RNA reduction in Cellulomonas is not as effective as it is for yeast, only a 50% reduction in RNA was obtainable.

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SECTION I

CONCLUSIONS

The work on single cell organisms has resulted in a general scheme for the production of protein isolates. This scheme, beginning with the cell menstrum (Scheme I) can be adjusted to produce either a crude protein fiber for animal feed or food grade protein. The processes involved are amenable to large scale operation and probably would be inexpensive enough to compete with protein isolates currently on the market.

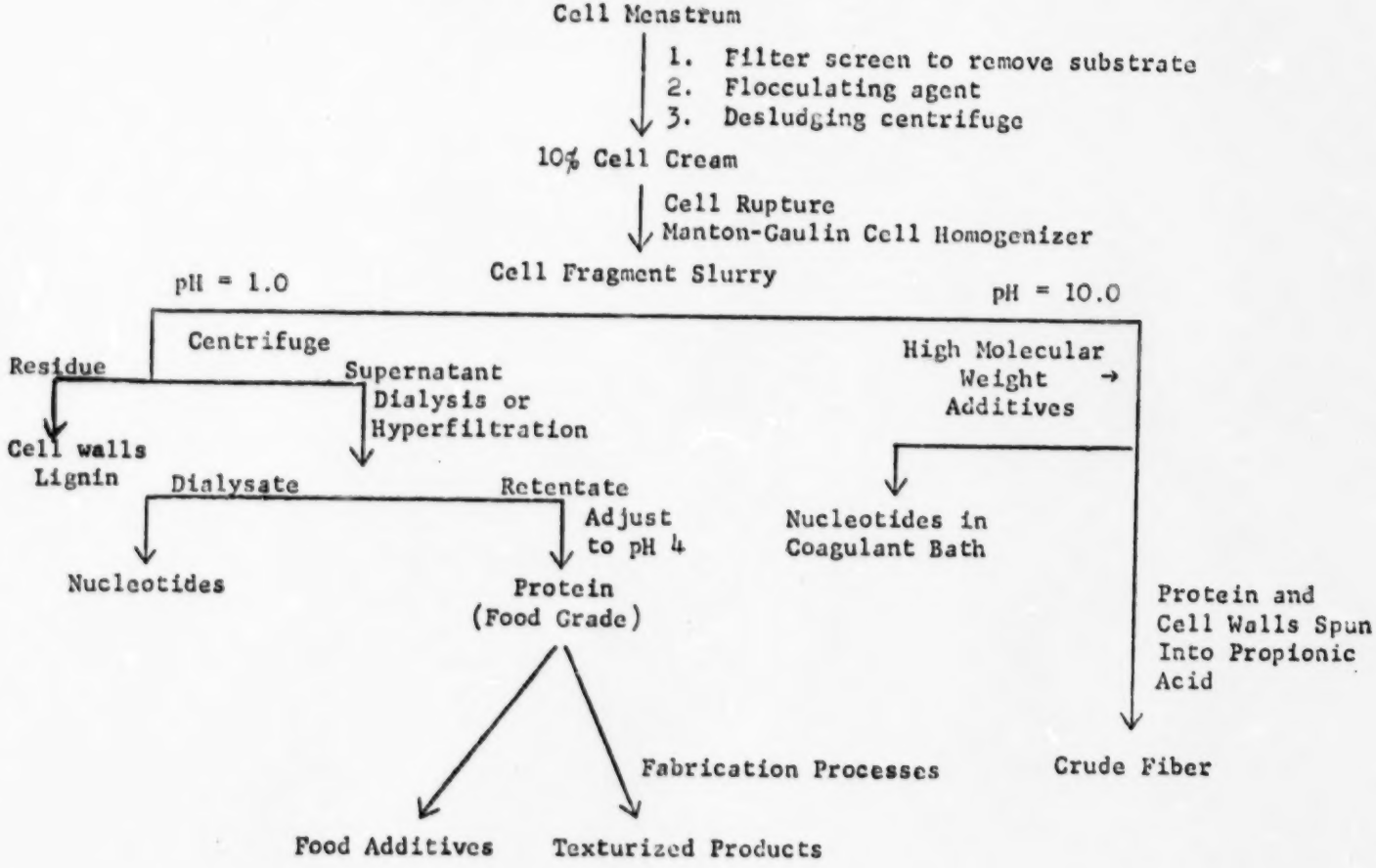
Preliminary studies employing flocculating agents for cell harvesting have demonstrated the feasibility of this technique but this phenomena is extremely complex and requires further investigation. The evolution of the food crisis into a world wide protein shortage prompted the realignment of one original aim of this research program. The investigation of non-food applications of microbial protein through suitable chemical modifications was deleted in favor of the more crucial problem of cell harvesting.

Assuming that a 10% cell cream can be obtained, a Manton-Gaulin cell homogenizer can be used to rupture the cell walls and produce a cell fragment slurry. Passage through the homogenizer removes or disintegrates any large particles which could clog a spinneret in the subsequent spinning processes so filtration of the spinning solutions will not be required. If crude fiber containing 55-60% protein is desired, one simply has to adjust the pH of the cell fragment slurry to 10.0, add a high molecular weight polymer to act as a fiber support, age the solution for four to six hours and then spin the protein into a propionic acid bath. Any organic or inorganic acid buffer can be used as a coagulant but propionic acid is non-toxic and digestable. The crude protein fiber can then be fabricated or modified to serve as an animal food or filler in an industrial application.

Food grade protein will require an additional purification step to remove the lignin and other dark colored impurities from the raw cells. Since these impurities are insoluble in acids, reducing the pH of the menstrum below the isoelectric point of the protein component precipitates the impurities and leaves a solution of nucleic acids and protein. Aging this solution for 10-12 hours effects the selective hydrolsis of the nucleic acids and produces a mixture of protein and nucleotides which can be separated by dialysis or hyperfiltration. Hyperfiltration would be preferred since the procedure also concentrates the protein solution. Finally, the protein can be precipitated by allowing the pH to rise to the isoelectric point. This procedure yields a cream colored powder containing 60-75% protein which could be employed as a food additive or fabricated to produce texturized protein products.

Scheme I

Protein Isolation from Single Cell Organisms



SECTION II

RECOMMENDATIONS

Research conducted under this grant was on a laboratory scale, since the Chemical Engineering department at Louisiana State University will be producing SCP on a pilot plant scale, it is recommended that large scale cell harvesting and protein purification also be investigated.

Continued analysis for trace metal content in bagasse and pesticide residues. Their effect on cell productivity and the amount of residue retained in the harvested SCP product should be studied. The possible use of urban solid wastes as the initial cellulose substrate would make this of particular importance since there is very little information on the trace metal content of recovered urban solid waste cellulose.

A study of the caustic wash liquor in terms of organics and BOD. Large scale SCP production will result in generation of extensive amounts of spent caustic wash liquor which was used to treat the cellulose substrate. Disposal of this liquor may require further treatment to prevent a possible pollution hazard.

Further studies in the area of cell harvesting should be conducted. The current emphasis for using SCP as animal feed presents the opportunity of using flocculating agents for cell harvesting that are not approved for human use.

SECTION III

INTRODUCTION

The current food shortages and continually rising prices have finally brought to light the critical food problems facing the entire world. Previously this problem was confined mainly to underdeveloped or "third-world" countries. The developed nations with their advanced technology were capable of not only feeding themselves but their surpluses could be used to feed the poorer countries. That hunger existed in the modern world was not due to food shortages but to economic, political, and sociological restraints. Now that true food shortages have become more global in scale, technology and research must keep pace to supply an expanding population with its necessary food requirements, especially protein. It is estimated that the world's food supply must be doubled by 1980 and tripled by the turn of the century to provide adequate food and nutrition to the increasing world population.[1] The solution to the problem is neither a single nor a simple one.

The proliferation of cellulosic wastes in urban and industrial areas as a result of increasing population density also presents a serious challenge to the technological development of our society. A similar increase in agricultural cellulosic wastes due to increased productivity and modified harvesting techniques is plaguing the rural areas. The magnitude of these problems is apparent when one considers that the United States alone produces more than 250 million tons of urban wastes per year[2], and cellulose comprises approximately 50% of the waste. Similarly, the U.S. Department of Agriculture has estimated that more than 200 million tons of cellulosic agricultural wastes[3] are produced each year and less than 1% of the wastes are utilized. The problem is compounded when centralized processing is required to isolate the crop; huge mountains of sugarcane bagasse, sugar beet vinasse, rice and wheat hulls, sawdust and many other cellulosics accumulate at these points and no satisfactory solution to the disposal of these wastes exists to date. More efficient utilization of cellulosic wastes would not only alleviate a severe waste disposal problem but also retard the depletion of our natural resources by our expanding population.

The Synthetic Foods Study Group at LSU is actively engaged in developing a microbial process for producing protein from cellulosic wastes. The results to date demonstrate that it is economically feasible to produce single cell organisms from bagasse[4] and these single cell organisms represent an excellent source of protein for both animal and human food supplements. The application of single cell protein as food supplements has been thoroughly demonstrated and many procedures for the production of "unconventional" protein foods have emerged.[5]

Current market prices for purified food and industrial grade protein are high and both the demand and prices have been rising rapidly during the last few years. The large volume of protein utilized in paper sizing, glue, film bases and emulsions, and food supplements has been supplied by soy protein isolate, gelatin, casein, whey, fish, bone meal and cottonseed isolates. Prices for these materials (usually containing from 85-95% proteins) range from 35 to 50 cents per pound and, because of rising prices, users have often had to change their protein supply to a lower cost source, i.e. from gelatine to soy protein isolate. Note that all of these resources are directly or indirectly related to agricultural products and thus are subject to the unstable market conditions normally encountered for these products. The production of single cell protein from solid wastes is based upon an ample supply of low cost raw material and controlled production costs so that we believe that SCP can compete strongly in the protein market. However, large scale production of protein extracts from a given single cell organism or mixture of organisms requires extensive research on the methods of extraction, purification and isolation of the protein component. Our efforts along these lines is the subject of this report.

Our research efforts during the past years have been directed toward two general objectives: thorough characterization of the bagasse substrate and development of a commercially feasible technique for removing the nucleic acids and isolating a pure protein extract from single cell organisms. Characterization of the bagasse substrate was required to ascertain the effect of the caustic pretreatment process utilized to increase the digestability of the bagasse by cellulytic organisms. Trace metal analysis by atomic absorption spectroscopy of the bagasse substrate was conducted. The use of gas chromatography as a possible means of analyzing the soluble sugar fractions in bagasse was also pursued.

An effective means of harvesting the bacterial cell from the culture media was also investigated. The small size of the bacteria, 0.3-0.5μ x 0.7-1.2μ, makes direct centrifugation of the culture media prohibitively expensive. The use of chemical additives which cause the cells to flocculate so they can be removed by a desludging centrifuge offers an inexpensive means of cell recovery. Several flocculating agents which are effective at a concentration of 0.1% are reported. The use of food grade proteins followed by pH adjustment offers an alternative to chemical flocculants. Casein acts as an excellent flocculating agent when the pH of the cell culture is adjusted from 7 to 5.

Methods for removal of the deleterious nucleic acid component, which is present in high concentrations in single cell organisms, have been evaluated and selective hydrolysis techniques have proved to be effective. Treatment of homogenized cells with 1.0N hydrochloric acid selectively hydrolyses the nucleic acids to low molecular

weight nucleotides which can be separated from the high molecular weight proteins by dialysis or hyperfiltration. The nucleic acid component of the purified single cell protein can be reduced from 10% to less than 2% using this technique. The use of an acid pH for nucleic acid removal also effects removal of the lignin impurity present in the cell culture. The acid treatment reduces the molecular weight and broadens the molecular weight distribution of the proteins, but reasonable mechanical properties are exhibited by the protein extract.

The nucleic acid content of the Cellulomonas can be reduced by a thermal shock followed by continued incubation of the cells to allow their endogenous ribonuclease to effect the hydrolysis but this procedure is not as effective as selective acid hydrolysis. The bacteria are given a heat-shock in the 65-75°C range for several seconds by means of a heat exchanger and then incubated for two hours at 50-55°C: the nucleotides dialyze from the intact cells. The process is highly effective for yeast, but reductions in RNA content of only 50% are observed with Cellulomonas.

Simultaneous removal of nucleic acids and fabrication of the protein into fibers by spinning the homogenized cells from an alkaline suspension into a dilute acid bath has been achieved. The spun protein fibers now have a texture which enable them to be incorporated into a variety of foods such as meat analogs.

SECTION IV

BAGASSE CHARACTERIZATION

The caustic pretreatment employed to activate the bagasse substrate has been standardized in terms of sodium hydroxide concentration (4%) treatment time (30 minutes), temperature (275°F) and steam pressure (30 psi).[6] Under these conditions, approximately 50% of the bagasse (based upon dry weight) is extracted by the alkali treatment and subsequent washings. Ethanolamine extraction of the remaining substrate indicates that this residue is greater than 90% pure α-cellulose and highly digestable. We conducted a detailed analysis of the initial bagasse substrate to determine the compositions of the components removed.

The bagasse was fractioned by a modification of the standard procedure described by Whistler and Gaillard.[7] Untreated bagasse was extracted with water, ethanol and benzene to remove the water soluble sugars and lipophilic component. After delignification with sodium chlorite, the bagasse was extracted with 4% sodium hydroxide under the pretreatment conditions. Polypropylene containers were utilized for the caustic extraction to prevent the incorporation of silica from glass vessels. The residue from this extraction was dried and recorded as the percent holocellulose based upon the initial dry weight of the bagasse.

The caustic solution contained the hemicellulose component which could be isolated by neutralization of the solution. To prevent contamination of the hemicellulose component by inorganic salts, the neutralization was conducted by charging the extract into a cellulose dialysis tube, suspending the tube in a dialysis bath and circulating the dialysate through a Dowex-50 column in the acid form until the pH of the dialysate remained at 5. After eight hours of dialysis, the contents of the tube was centrifuged; the residue was dried and weighed to determine the hemicellulose I content.

The supernatant from the centrifugation was concentrated to approximately 100 ml and this solution was added dropwise to 500 ml of ethanol. The precipitate was isolated by centrifugation, dried, weighed and recorded at the hemicellulose II content. The supernatant was evaporated to dryness and the residue was considered hemicellulose III. The results of a complete duplicate analysis are summarized in Table I.

TABLE I

Composition of Bagasse

Component	Sample I Wt %	Sample II Wt %
Water Soluble	10.1	11.3
Alcohol + Benzene Soluble	1.1	0.8
Lignin Con ent	9.2	10.3
α-Cellulose	47.2	48.3
Hemicellulose I	12.5	11.9
Hemicellulose II	7.3	6.8
Hemicellulose III	15.2	14.7

These results indicate that the caustic treatment is indeed removing the hemicellulose and lignin fraction of the bagasse. The α-cellulose content of the treated and washed samples as estimated by ethanolamine extraction increases to greater than 90% so the treatment process is a purification step as well as an activation process.

The composition of the wash liquor was briefly examined. The pH of the liquor was 9.4 indicating that most of the caustic is actually consumed in the treatment process. Since hemicelluloses are known to degrade under highly alkaline conditions to produce xyloisosaccharinic acid,[8] the alkali consumption can be attributed to the formation of weak acid salts. Neutralization of the alkaline extract to pH 7 produces a flocculant black precipitate which is 30-35% ash even after careful washing. The precipitate consists of the lignin component and most of the hemicelluloses; both of these components are polyfunctional weak acids and serve as cation binders. This is a very useful property in view of the trace metals normally found in raw bagasse. The wash liquor can be clarified easily by addition of calcium salts such as $CaCl_2$ or lime.

The trace metal content of the bagasse was investigated to determine if the metals were present in concentrations high enough to influence the fermentation process or to contaminate the single cell product. The bagasse was digested with 70% nitric acid, followed by 35% perchloric acid to completely oxidize all organic material.

The supernatant liquid was then analysed by atomic absorption using an acetylene-air burner. The results from this analysis are summarized in Table II.

TABLE II

Trace Metal Content of Bagasse

Trace Metal	Untreated Bagasse, PPM	Treated Bagasse, PPM
Cu	23	1.2
Cr	~0.1	---
Pb	83.7	11.3
Cd	15.6	---
Zn	18.2	---
Mg	15.7	---
Mn	19.2	---
Ca	21.9	---
As	49.2	9.8
Pb	48.7	15.6
Pb	22.9	10.0

The most significant trace metals are lead, arsenic and copper but the treatment process reduces the concentration of these contaminates to acceptable levels. These results are subject to variation depending upon the source and history of the bagasse substrate. Further monitoring of the lead and arsenic content will be conducted to establish a reasonable norm for these contaminents.

We have standardized a technique for analysing soluble carbohydrates by gas chromatography and are currently determining the nature and composition of the soluble carbohydrates in the menstrum. Our preliminary results revealed that the phenol-sulfuric acid procedure for soluble carbohydrates, which was used in the early stages of the single cell project, did not always correlate well with the actual sugar content. More accurate results were obtained by passing the menstrum through an ion-exchange column, freeze-drying the eluate and silylating the residue with a mixture of trimethylchlorosilane and hexamethyldisilazane dissolved in pyridine. The composition of the resultant silyated sugar mixture was determined by temperature programmed gas chromatography on a silicone

gum column using inositol as a standard. Intensive investigation of the highly complex mixtures of mono- and poly-saccharides by gas chromatography has indicated that quantitative and reproducible analyses of the sugars is not possible at this time.

SECTION V

EVALUATION OF ANALYTICAL TECHNIQUES

A primary objective of the project is to isolate the protein component of the single cell organisms in a pure form. A preliminary survey of techniques for protein isolation quickly demonstrated the necessity for developing accurate and precise analytical procedures for protein determination. Further, a routine method for assaying the composition of the harvested cultures was required since analysis revealed that the protein produced in a pilot plant consisted of a mixture of cellulose, soluble carbohydrates, lignin, and inorganic salts in addition to the whole cells. The amount of each impurity varied from culture to culture, depending upon the harvesting and drying techniques employed.

Several different analytical procedures for quantitative determination of protein composition were investigated. The procedures were evaluated by the following criteria: accuracy on heterogeneous protein mixtures, interferences from the impurities present, precision, and time required for analysis. The common spectrophotometric techniques proved to be both inaccurate and relatively imprecise when applied to whole cell mixtures. The Biuret[9] test yielded consistently high results when compared with the protein composition obtained from total amino acid analysis. The Lowry[10] method was consistently equally unsatisfactory; the results were consistently low and dependent upon the nature of cell isolation technique. Calculation of the protein composition from the nitrogen content was extremely inaccurate; protein content was overestimated by as much as 20% due to the presence of nucleic acids and nutrient salts. Finally, the ninhydrin test was found to yield satisfactory results although the procedure required preliminary hydrolysis of the protein to the free amino acids. The hydrolysis was effected by treating the sample with 6N HCl at 145°C in a sealed tube under a nitrogen atmosphere for four hours. Ninhydrin was developed from the amino acid mixture according to the procedure of Moore and Stein.[11] A comparison of the testing procedures is summarized in Table III.

TABLE III

Methods for Estimating Protein Content

Sample	Amino Acid Analysis	Ninhydrin Test	% Nitrogen Determination	Lowry Method
Casein	93.5%	95%	95.3%	94%
Lactalbumin	---	80%	78.9%	---
Whole Cell Fractions				
1	45.5%	---	74.3%	41%
2	51.6%	---	64.9%	38%
3	46.6%	48%	64.9%	38%
4	78.6%	80%	---	---
5	73.5%	75%	---	---

The ninhydrin test always gave results that were within $\pm$ 5% of the protein content by amino acid analysis. Its one disadvantage was that it is not as rapid a test as the Biuret or Lowry method since the protein must be hydrolyzed to its free amino acids before analysis. The slow decomposition of the ninhydrin test solutions required that a standard sample also be analyzed each time the test was used. The rapid procedure of the Biuret and Lowry methods make them ideal for use when qualitative protein analysis is required, but their use as a quantitative procedure in SCP analysis is not recommended.

The ninhydrin procedure was also used to correlate the Klett Procedure for measuring cell density with actual protein content. An aliquot of the culture media was filtered through a millipore screen to remove the bagasse particles and then diluted with two volumes of ethanol. The coagulated protein and the single cell mixture was isolated by centrifugation, dried and analyzed for protein content. There is a reasonable correlation between Klett units and protein content for the initial phases of growth, but a significant deviation occurs when cell densities exceed 3-4 g/l or the culture has been allowed to age. The deviations probably result from the tendency of the cells to undergo autolysis upon aging which releases extracellular protein and produces agglommeration. Fortunately, these errors tend to make Klett measurements a conservative estimate of the protein content, but accurate estimation of the protein content must be conducted by the ninhydrin procedure.

The first step in the ninhydrin technique, acid hydrolysis is also the initial step required for the analysis of the protein amino acid composition by gas chromatography. The acid hydrolysis procedure is as follows:

Between 25.00 and 50.00 mg of dry sample is weighed into a protein hydrolysis tube, (Kontes, K-89685, 25 ml) 10 ml of 6N HCl is then added. The tube is flushed with nitrogen and sealed. It is then heated in an oil bath at 145 ± 2°C for 4 hours. After heating the tube is cooled in an ice bath before opening. The sample is transferred quantitatively to a 250 ml volumetric flask and diluted to volume with distilled water. A 1 ml aliquot is used for the Ninhydrin Test[11] with hydrolyzed casein as the standard (0.015 mg/ml). Since the Ninhydrin Test solutions are not stable the standard must be analyzed each time the test is conducted.

Amino acid analysis by gas chromatography[12] requires that the hydrolyzed protein solution be treated in a different manner. When the hydrolysis tube is opened after heating for 4 hours, 1.0 ml of an internal standard is added (DL-α, ε-Diaminopimelic acid, 2.5 mole/ml). This solution is then passed through a cation exchange column (Amberlite, IR-120) after the method of Gehrke *et al.*[13] The eluted sample is approximately 15 ml in volume. A 3 ml sample of this elutant is transferred to a 12 x 75 mm culture tube with a Teflon lined screw cap. The sample is dried by a stream of nitrogen while heating the tube in an oil bath at 100 ± 2° for ten minutes. Once dry, 0.4 ml of 8N HCl in anhydrous propanol is added to the tube which is then sealed with the teflon lined cap. The sealed tube is heated at 100± 2°C for ten minutes, cooled to room temperature, and the top removed. The sample is dried as before being careful to take it just to dryness. The entire procedure is repeated starting with the addition of the 0.4 ml of 8N HCl in anhydrous propanol. After the sample is dried a second time, 0.1 ml of ethyl acetate is added followed by 0.2 ml heptafluorobutyric anhydride (HFBA). The tube is resealed and heated at 150 ± 2°C for 10 minutes. It is allowed to cool to room temperature and then the sample is dried by a stream of nitrogen at room temperature. Just at reaching dryness, exactly 0.1 ml of ethyl acetate is added and 3 μl of this solution is injected into the gas chromatograph. The derivatives tend to decompose after approximately one week of storage at -5°C.

Satisfactory separation of the individual amino acid derivatives is obtained on a 12 feet, glass column of 3% OV-1 on Chromasorb-W using direct on column injection. The column is maintained at 100°C for 4 minutes after sample injection and then temperature-programmed to 250°C at 4°C/min. Helium was used as the carrier gas at a flow rate of 40 cc/min. Shown in Figure 1 is a chromatogram of an amino acid standard mixture containing 0.015 μ mole of each amino acid.

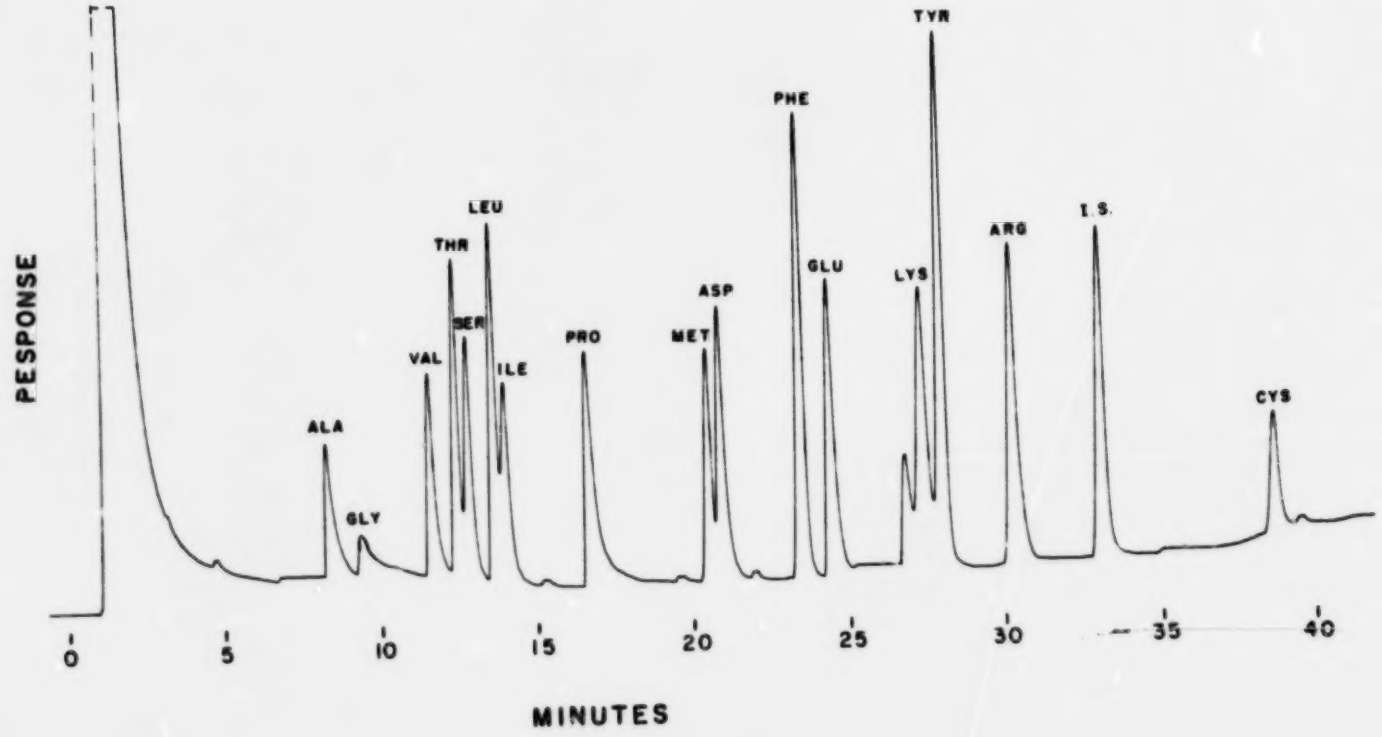


Figure 1. Gas Chromatogram of the N-HFB n-propyl derivatives of a standard amino acid mixture on a 3% OV-1 column. Each peak represents 0.015 umole of amino acid.

A Hewlett-Packard model 7123A recorder and model 3373B integrator was used to record the data. The calculations were done by computer and were based on the following equations:

$$RMR_{a.a./I.S.} = \frac{\left[A_{a.a./moles\ a.a.}\right]}{\left[A_{I.S./moles\ I.S.}\right]}$$

RMR = relative molar response

a.a. = amino acid

I.S. = internal standard

$A_{a.a.}$ = area of amino acid peak on chromatogram

$$Mole\ \% = \frac{\frac{A_{a.a.}}{RMR_{a.a./I.S.}}}{\sum \frac{A_{a.a.}}{RMR_{a.a./I.S.}}} \times 100$$

$$g_{a.a.} = \frac{A_{a.a.} \times GFW_{a.a.} \times g_{I.S.}}{A_{I.S.} \times GFW_{I.S.} \times RMR_{a.a./I.S.}}$$

$$w/w\ \%\ of\ amino\ acid = \frac{g_{a.a.}}{grams\ sample} \times 100$$

TABLE IV

RMR of Amino Acid Standards

Amino Acid	Rentention Time (Min)	$RMR_{a.a./I.S.}$
Ala	8.7	0.55
Gly	9.8	0.28
Val	12.0	0.59
Thr	12.8	0.76
Ser	13.2	0.64
Leu	14.0	0.89
Ile	14.4	0.62
Pro	17.0	0.90
Met	20.9	0.60
Asp	21.3	0.83
Phe	23.9	1.30
Ghi	24.9	1.02
Lys	27.8	1.03
Tyr	28.5	1.55
Arg	30.7	1.01
Cys	39.2	0.68
I.S.(DL-α, ε-Diaminopimelic acid)	33.6	1.00

In Table IV is the RMR of the amino acids with DL-α, ε-Diaminopimelic Acid as the I. S. This offers the most desirable type of protein analysis, a complete amino acid analysis. The quantitative analysis of amino acids by gas chromatography requires the use of an internal standard (I.S.) if the procedure is to be both rapid and accurate. The initial procedure used β-alanine. Ornithine, Norvaline, steric acid, α-amino n-butyric, β-amino n-butyric and p-amino benzoic acid were all investigated as possible I.S. but were eliminated due to a retention time corresponding to a naturally occurring amino acid. β-alanine has a retention time between glycine and valine. However as the OV-1 column lost resolving power with extended use, it became increasingly difficult to separate the β-alanine and valine peaks. A further search of possible I.S.

led to the selection of DL-α, ε-Diaminopimelic acid as the I.S. of choice.

The analysis of an actual SCP sample requires that the hydrolyzed protein sample be put through a cation exchange column before derivation to remove interfering impurities. The use of an I.S. with both an amine and a carboxcylic acid group enables the compound to be added to the hydrolyzed protein solution immediately after hydrolysis. Then an ion exchange clean up of the sample and subsequent dilution or concentration will not have any effect on the calculations for amino acid content.

SECTION VI

METHODS OF PROTEIN EXTRACTION

An alkine buffer extraction technique was used to produce protein concentrations from Cellulomonas grown on purified wood pulp as follows:

Sample #1

A 10% suspension of Cellulomonas in bicarbonate buffer,[14] pH = 9.7, was prepared. The cell walls were disrupted and the solution centrifuged to remove them. The cell wall fraction was again extracted with buffer and the two extracts combined. The protein was precipitated by the addition of acid to obtain a pH of 4.0. This protein precipitate was centrifuged to remove it from solution, suspended in distilled water and dialyzed to a neutral pH. The amino acid analysis of the product is in Table V.

Sample #2

This sample was prepared in a similar manner. After precipitation and centrifugation of the protein, the residue was extracted with the following organic solvents to remove the lipids and any organic soluble impurities, ethanol, acetone, chloroform-methanol 2:1, and ether in that order.

Sample #3

A portion of sample #2 was redissolved in buffer and $MnCl_2$ was used to precipitate the nucleic acids from the solution. After addition of the $MnCl_2$ the precipitate was centrifuged and removed. The protein solution was then precipitated by acid as in sample #1 and finally dialyzed. A test of the $MnCl_2$ precipitate by the Biuret reagent[88] indicated some protein was present. All of the samples were freeze dried prior to analysis.

TABLE V

Alkaline Buffer extracts of Disrupted Cellulomonas Cells

Amino Acid	Sample #1 Percent of Sample	Sample #2 Percent of Sample	Sample #3 Percent of Sample
Lysine	3.18	3.34	2.90
Histidine	1.24	1.40	1.22
Arginine	4.58	4.68	4.02
Aspartic acid	4.51	4.89	4.12
Threonine	2.35	2.82	2.44
Serine	1.44	1.82	1.49
Glutamic acid	6.88	7.89	6.84
Proline	1.70	1.88	1.76
Glycine	2.72	2.97	2.76
Alanine	5.36	5.84	5.50
Valine	3.71	3.90	3.62
Methionine	0.64	0.88	0.72
Isoleucine	1.71	1.94	1.62
Leucine	3.76	4.23	3.72
Tyrosine	1.04	1.21	1.05
Phenylalanine	1.78	1.94	1.72
Total Amino Acid	46.60	51.63	45.50

The use of an alkaline buffer solution to solubilize and extract proteins is a well known technique in protein chemistry. This procedure was used to form various protein extracts from Cellulomonas and Alcaligenes cells. The previous section outlines the experimental procedure used and Table V contains the results of analyses of the typical products obtained. The protein content of the initial extract (Sample #1) can be increased slightly by removing the lipids and organic soluble material (Sample #2). However, it is doubtful if this step is necessary and would ever be incorporated into a SCP process since the presence of lipids in the product would only lower the protein content by a small percentage and would have no adverse effects. The use of $MnCl_2$ as an agent

for nucleic acid removal was unsuccessful in the sense that some of the protein was precipitated along with the nucleic acids. This $MnCl_2$ precipitate gave a positive test to the Biuret reagent and the amino acid analysis of the product (Sample #3) indicated that the protein content had dropped by 6% from Sample #2. Also the $MnCl_2$ reagent remained to some extent in the protein product which was very undesirable.

The alkaline buffer extractions of proteins from Cellulomonas were repeated using samples grown on alkali treated bagasse on a pilot plant scale. These samples were characterized by high salt, lignin, and cellulose content with a low protein content as compared to samples grown on a laboratory scale on purified cellulose substrates which were characterized by low salt, lignin, and cellulose content with a high protein content. Table VI gives the composition of the original SCP product. The whole cells were extracted first to obtain external protein (Fraction A). Fraction B was obtained from the cells after the cell walls were ruptured, and Fraction C was the protein extracted from the cell walls. The ratio of the protein yield from each fraction was 1:3:0.5 for Fraction A, B, and C respectively. The amino acid composition of these fractions are contained in Table VII. The very low protein content of these samples was due to the presence of large amounts of impurities. The results indicate that there is a substantial amount of extracellular protein present (Fraction A) in the harvested SCP product. The cells themselves yielded three times the extracellular protein (Fraction B) and one-half the amount of the extracellular protein could be extracted from the cell walls (Fraction C). If optimum protein yields are to be obtained from a fermentation media, the initial harvesting of the cells should be carefully monitored to prevent any protein loss through disposal of the culture media. If the cells are harvested by centrifugation an adjustment of the pH to between 4.0 to 4.7 will result in total removal of the cells and protein from the culture media. The protein extracted from the cell walls (Fraction C) is also an important part of the SCP product. Since an extra step to extract this protein would be costly, inclusion of this protein in the SCP product can be assured by deleting a step to remove the cell walls themselves.

The use of an alkaline buffer to obtain a protein extract also extracts the nucleic acids and these must be removed later. Lignin is also very soluble in an alkaline media and would be incorporated into the protein fraction. Lignin has been isolated from a harvested Cellulomonas culture.

Confirmation that bagasse lignin is one of the major impurities present in harvested Cellulomonas grown on alkali treated bagasse was obtained in the following manner. Thirty grams of drum dried Cellulomonas grown on alkali treated sugar cane bagasse was extracted with 95% ethanol in a Soxhlet Extractor until the extracting

TABLE VI

Cellulomonas Grown on Alkali Treated Bagasse

Amino Acid	Percent of Sample	Percent of Total Peptide A.A.
Lysine	2.33	7.87
Histidine	0.86	2.91
Arginine	2.20	7.43
Aspartic acid	2.80	9.46
Threonine	1.54	5.20
Serine	1.04	3.51
Glutamic acid	3.67	12.40
Proline	1.08	3.65
Glycine	1.82	6.15
Alanine	3.18	10.74
Valine	2.16	7.30
Methionine	0.64	2.16
Isoleucine	1.32	4.46
Leucine	2.89	9.76
Tyrosine	0.81	2.74
Phenylalanine	1.26	4.26
Total Amino Acid	29.60	100.00

TABLE VII

Protein Extracts from Cellulomonas Grown on Bagasse

Amino Acid	Extract from Whole Cells: Fraction A % of Sample	Extract from Whole Cells: Fraction A Total % of Peptide A.A.	Extract from Disintegrated Cells: Fraction B % of Sample	Extract from Disintegrated Cells: Fraction B Total % of Peptide A.A.	Residual Protein Extract: Fraction C % of Sample	Residual Protein Extract: Fraction C Total % of Peptide A.A.
Lysine	0.58	7.70	2.11	7.78	1.19	8.08
Histidine	0.17	2.26	0.64	2.36	0.46	3.12
Arginine	0.36	4.78	1.32	4.87	0.70	4.75
Aspartic acid	1.03	13.67	3.78	13.94	2.06	13.99
Threonine	0.41	5.44	1.77	6.53	0.94	5.70
Serine	0.30	3.98	1.38	5.09	0.74	5.02
Glutamic acid	1.07	14.21	3.28	12.09	1.78	12.08
Proline	0.24	3.18	0.86	3.17	0.47	3.19
Glycine	0.86	11.42	1.62	5.97	0.92	6.25
Alanine	0.58	7.70	2.03	7.48	1.12	7.60
Valine	0.42	5.58	1.84	6.78	0.94	6.38
Methionine	0.07	0.93	0.27	0.99	0.26	1.77
Isoleucine	0.35	4.65	1.48	5.46	0.78	5.29
Leucine	0.64	8.50	2.64	9.73	1.31	8.89
Tyrosine	0.18	2.39	0.79	2.91	0.42	2.85
Phenylalanine	0.32	4.25	1.31	4.83	0.64	4.34
Total Amino Acid	7.53	100.64	27.12	99.98	14.73	99.30

ethanol was clear. The resulting brown solution was filtered and evaporated with a rota-vac. The brown oily residue was washed with cold water, ether and dried again under vacuum. The material was dissolved in dioxane, filtered and precipitated into cold water. The precipitate was freeze dried to yield 0.66 g of a medium brown powder. The infrared spectrum is consistent with that expected for pure bagasse lignin. The ultraviolet spectra had a maximum at 330 nm compared to a UV maximum of 315 nm for enzymatically released bagasse lignin.[15]

According to DeStevens and Nord[15] enzyme action on cellulose releases chemically bond lignin. In the alkali pretreatment of the bagasse large amounts of lignin are removed, however, as the Cellulomonas degrade the cellulose further the remaining lignin will be released. This lignin is acid insoluble and is precipitated along with the cells if a pH adjustment to 4.0 to 4.7 is used to harvest the cells before centrifugation. The lignin is then extracted with the protein and gives it a dark brown color.

The use of an acid extraction scheme was also investigated as a means of obtaining a protein concentrate in the same manner that alkaline buffers are used. This would be a means of producing a protein concentrate free from nucleic acids and lignin. A 5% solution of disrupted Cellulomonas grown on purified wood pulp was extracted in 0.1N HCl at 25°C for 10 minutes. The residue was centrifuged and the extraction procedure was repeated twice. The three extracts were combined, dialyzed to a neutral pH and freeze dried. Of the starting material 51.2% was extracted into the acid, 38.2% remained in the cell wall fraction and the remainder was lost during dialysis. The amino acid analysis of the acid extract is shown in Table VIII.

TABLE VIII

Protein Extract from Cellulomonas Using 0.1N HCl at 25°C

Amino Acid	Percent of Sample	Percent of Total Peptide A.A.
Lysine	2.32	5.53
Histidine	0.93	2.21
Arginine	3.10	7.38
Aspartic acid	4.24	10.10
Threonine	0.62	1.48
Serine	1.33	3.17
Glutamic acid	6.66	15.86
Proline	2.35	5.60
Glycine	2.93	6.98
Alanine	5.05	12.03
Valine	3.68	8.76
Methionine	0.87	2.07
Isoleucine	1.91	4.55
Leucine	3.58	8.52
Tyrosine	0.87	2.07
Phenylalanine	1.55	3.69
Total Amino Acid	41.99	100.00

To improve the yield of the acid extraction technique a longer incubation time was investigated. A symbiotic culture of Cellulomonas and Alcaligenes was used. The amino acid composition of this material is in Table IX.

TABLE IX

Symbiotic Culture of Cellulomonas and Alcaligenes

Amino Acid	Percent of Sample	Percent of Total Peptide A.A.
Lysine	4.67	6.87
Histidine	2.06	3.03
Arginine	6.24	9.16
Aspartic acid	6.37	9.37
Threonine	3.22	4.74
Serine	2.44	3.59
Glutamic acid	9.57	14.09
Proline	1.88	2.77
Glycine	4.23	6.23
Alanine	6.31	9.29
Valine	5.16	7.60
Methionine	1.70	2.50
Isoleucine	3.35	4.93
Leucine	5.68	8.36
Tyrosine	2.05	3.02
Phenylalanine	3.00	4.42
Total Amino Acid	67.93	99.97

By increasing the time and temperature of the reaction, the nucleic acids could also be completely hydrolyzed. The cells were suspended (5% concentration in) dilute acid for 20 hours at 37°C. They were then centrifuged and the residue extracted twice with a volume of dilute acid equal to half the original sample volume. The extracts were combined, dialyzed to a neutral pH and finally freeze dried.

TABLE X

Acid Extracts from Symbiotic Culture of

Cellulomonas and Alcaligenes

HCl* Concentration	% Material Recovered from Acid Extract	% Material in Cell Wall Residue	% Material Loss During Dialysis
0.1 N	78.9	6.5	14.6
0.2 N	73.2	6.8	20.0
0.3 N	74.9	4.5	20.7
0.4 N	70.0	5.6	24.4

*20 hours at 37°C

Table X has the results on product yield using different acid concentrations for protein extraction. The longer extraction times, 20 hours versus 10 minutes resulted in an increase in protein yields of 20% to 30%. The much longer times enabled more protein to be removed from the cell walls. They also resulted in more sample being lost during the dialysis step. However, not all of the sample lost is protein, the diffusate in all of these experiments was monitored by UV analysis which indicated that nucleic acid components were being lost.

SECTION VII

NUCLEIC ACID REMOVAL BY ACID HYDROLYSIS

The effect of acid hydrolysis for the removal of nucleic acids from pure Alcaligenes cells was investigated. The amino acid analysis of the starting material is shown in Table XI. The

TABLE XI

Alcaligenes Cells

Amino Acid	Percent of Sample	Percent of Total Peptide A.A.
Lysine	4.72	6.57
Histidine	2.01	2.81
Arginine	5.74	8.01
Aspartic acid	7.53	10.52
Threonine	3.50	4.89
Serine	2.50	3.62
Glutamic acid	9.67	13.51
Proline	1.85	2.59
Glycine	4.25	5.90
Alanine	6.10	8.52
Valine	5.61	7.84
Methionine	2.10	2.93
Isoleucine	4.00	5.59
Leucine	6.16	8.61
Tyrosine	2.37	3.31
Phenylalanine	3.50	4.89
Total Amino Acid	71.68	100.11

removal of the cell walls was deleted. The hydrolysis was conducted on the entire cell components after homogenation. A 5% suspension was hydrolyzed for 12 hours at 37°C. in dilute HCl and then dialyzed for 12 hours. The results of two typical runs are in Table XII.

TABLE XII

Acid Hydrolysis of Alcaligene at 37°C for 12 Hours

HCl Concentration	% Recovery of Starting Material	% Protein of Recovered Product	% Nucleic Acids Removed
0.5 N	84	71	88
0.5 N*	86	72	69
1.0 N	80	73	88
1.0 N*	80	72	85

*Acetylated dialysis membrane used in dialysis

Using shorter hydrolysis times at higher acid strengths it was possible to recover greater than 80% of the starting material (Table XII). The protein content of the product obtained is essentially that of the starting material. This would indicate that only the nucleic acid components and a small amount of low molecular weight proteins are lost since no attempt was made to remove the cell wall fraction.

It was thought that the use of a dialysis membrane with the pore size reduced through acetylation would prevent some protein loss; however, there is very little difference between the two types of membranes using 0.5N HCl (Table XII). The amount of nucleic acids lost is more noticeable. From these results it would appear that at a concentration of 1.0N HCl the nucleic acids are almost completely hydrolyzed to low molecular weight components which are lost on dialysis. In 0.5N HCl there must be higher molecular weight nucleic acid fragments remaining, for when the pore size of the dialysis membrane is decreased the amount of nucleic acid reduction also decreases.

A major problem in utilizing SCP as a possible protein source for human consumption is its high nucleic acid content. Therefore

this technique was thoroughly investigated as a method to reduce the nucleic acid content to an acceptable level.

Using pure RNA and DNA samples the effect of a mild acid treatment was investigated. Yeast RNA was treated with 0.5 and 1.0N HCl at 37°C for 12 hours. A sample in distilled water was used as the control. The initial nucleic acid concentration was 200 mg/ml. The samples were then dialyzed for 12.5 hours against distilled water. At the end of the dialysis, 91% of the RNA had diffused through the dialysis membrane. Treatment of DNA under similar conditions produced a 50% reduction in the DNA content (Figure 2). The use of pure RNA and DNA made it possible to analyze the difusate accurately at 260 nm in the UV. Cell cultures grown on bagasse contain impurities which make this type of analysis extremely difficult. The nucleic acid samples in the dialysis membranes were suspended in a 2 l container in which the water was changed hourly. The UV absorption of this solution was recorded and at the end of the dialysis time the total concentration of diffused nucleic acids components was compared to the amount retained within the dialysis membrane. The results agree with ± 2%. The same procedure was used to study the effect on Alcaligenes cells in Figure 3 and 4 with Figure 5 indicating the effect of acid concentration on nucleic acid removal.

The potential of using acid hydrolysis for nucleic acid removal has been demonstrated by hydrolysing pure RNA and DNA. Selective hydrolysis has also been applied successfully to the purification of single cell protein from a Cellulomonas-Alcaligenes cell cream as well as pure cell cultures grown on non-cellulosic media. The cells are passed through a Manton-Gaulin Cell Homogenizer as a 10% suspension to rupture the cell walls and then freeze-dried in order to obtain quantititive recovery data. Samples grown on bagasse were dissolved in 1.0N HCl and hydrolyzed for several hours. They can be centrifuged to remove the larger cell wall fragments and acid insoluble impurities. The protein remains dissolved in the supernatant because the pH is below the isoelectric point. This solution is nearly colorless since the dark-colored, acid-insoluble lignin can be removed by the contrifugation step. The protein from some Alcaligenes cell cultures grown on different media failed to completely dissolve in 1.0N HCl due to variations in the cell harvesting techniques, but the nucleic acids in the cell suspension appeared to hydrolyse as rapidly as those in a solution. The homogenized cells were allowed to stand at 37°C for 10-12 hours to assure maximum hydrolysis of the nucleic acid component. Longer hydrolysis times failed to increase the amount of mononucleotides extracted to any significant extent. Raising the hydrolysis temperature to 50°C did not reduce the reaction time enough to compensate for the accelerated hydrolysis of the protein component at higher temperature.

Following the hydrolysis step, the protein solution or suspension is charged into a seamless regenerated cellulose dialysis tube and dialyzed with distilled water to remove the residual nutrient salts and the mononucleotides. The dialysis step does not appear to be affected by the physical state of the protein extract; equally good results were obtained on either protein solutions or suspensions. Since the Alcaligenes cells were completely free of colored contaminants, more precise analytical data could be obtained by ultraviolet spectroscopy and these cell cultures were used to optimize the experimental conditions for nucleic acid removal. Approximately one-half of the initial nucleic acid content can be removed within four hours regardless of the method of pretreatment (Figure 4). However, further diffusion of mononucleotides is dependent upon the concentration of hydrochloric acid employed in the hydrolysis step. In general, the residual nucleic acid content was less than 15% of the original content if the hydrolysis media was at least 1.0N in hydrochloric acid. Higher acid concentrations (Figure 5) failed to yield a significant improvement in the nucleic acid removal and promoted a more rapid hydrolysis of the protein. The dialysis process appeared to be complete after twelve hours, but an acceptable level of the overall nucleic acid content of the protein isolate could be obtained in 4-6 hours. Addition of ethylene diamine tetraacetic acid (EDTA) appears to be as effective as 0.5N KOH and 1N HCl in reducing the nucleic acid content (Figure 3).

When the dialysis step is completed, the protein can be isolated by freeze-drying the retentate. The product is a tasteless, odorless cream-colored flour which contains approximately 50-75% protein and 2-4% nucleic acids. Some low molecular weight proteins are lost in the diffusion step, but 80% of the starting material is recovered regardless of the severity of the hydrolysis step (Table XIII). The acid hydrolysis does not appear to increase the percentage of diffusable cell components other than nucleic acids. The protein content of the isolate is essentially that of the initial cell suspension since no effort was made to remove the cell wall fragments in this study.

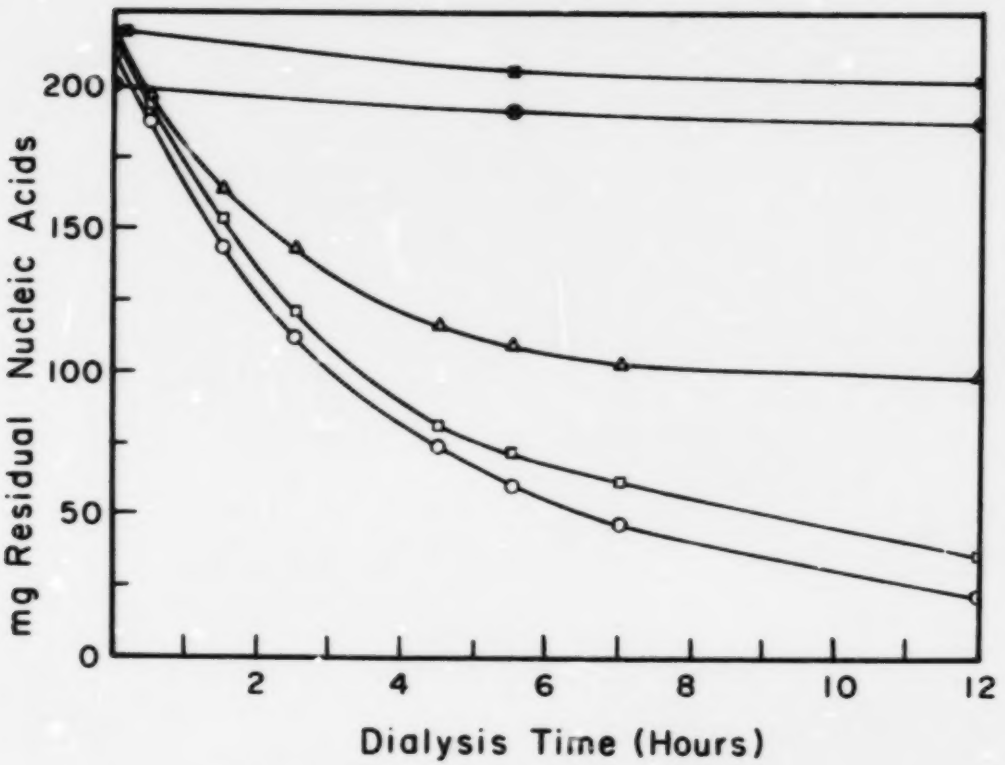


Figure 2. Effect of acid hydrolysis upon the diffusion of nucleotides through a regenerated cellulose dialysis tube. Initial nucleic acid concentration, 200 mg/ml; hydrolysis time, 12 hr., hydrolysis temp., 37°C. Hydrolysis medium and substrate: ■, DNA in H_2O; Δ, DNA in 1.0N HCl; ●, RNA in H_2O, □, RNA in 0.5N HCl, ○, RNA in 1.0N HCl.

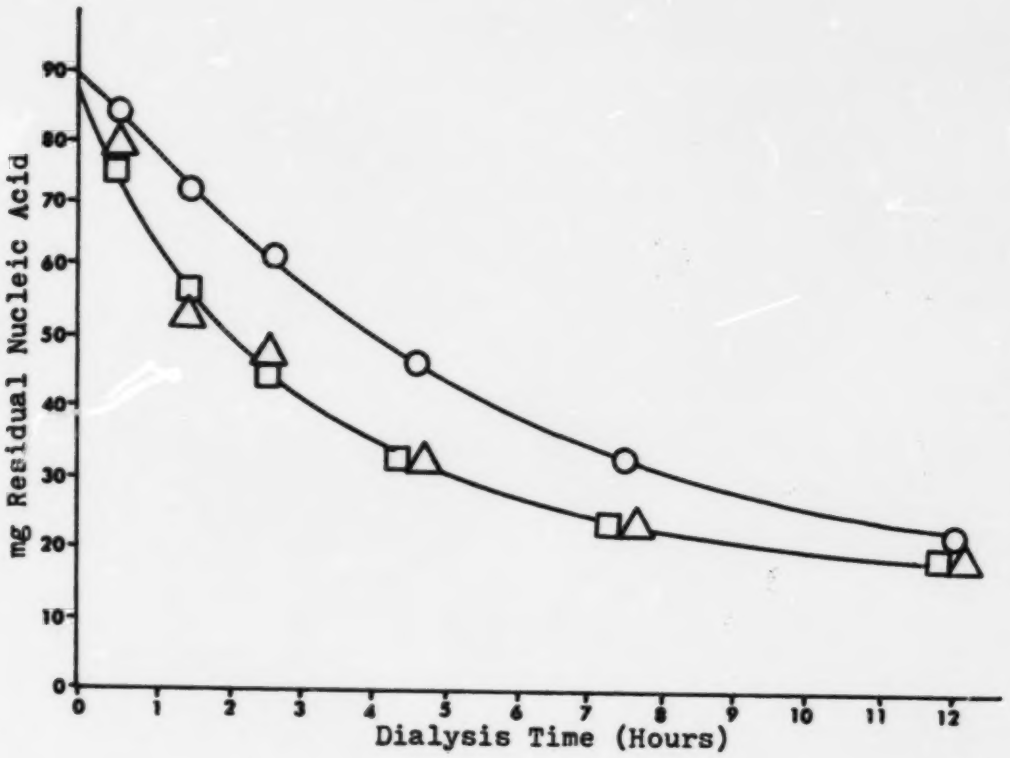


Figure 3. Removal of nucleotides by dialysis of selectively hydrolysed Alcaligenes. Initial nucleic acid concentration, 90 mg/ml; hydrolysis time, 12 hr.; hydrolysis temperature, 37°C. Hydrolysis medium: ○, 0.5N KOH; □, 0.01 EDTA; △, 1.0N HCl.

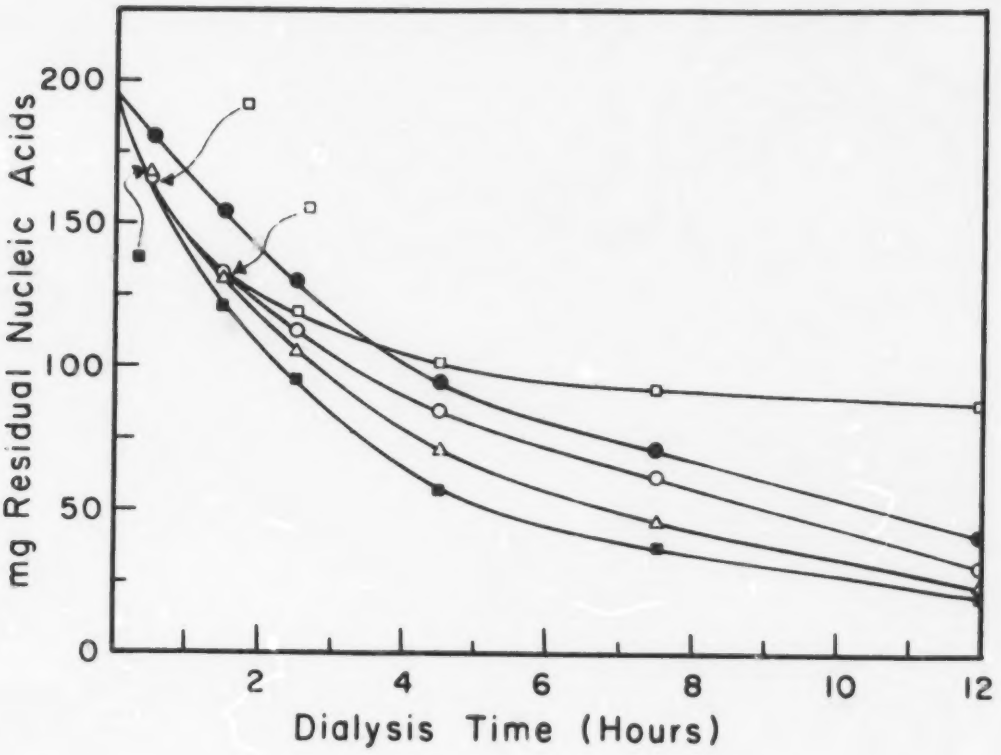


Figure 4. Removal of nucleotides by dialysis of selectively hydrolysed Alcaligenes. Initial cell concentration, 50 mg/ml; hydrolysis time, 12 hr.; hydrolysis temp., 37°C.; hydrolysis medium: □, No hydrolysis; ●, 0.5N KOH; ○, 0.5N HCl; △, 1.0N HCl; ■, 3.0N HCl.

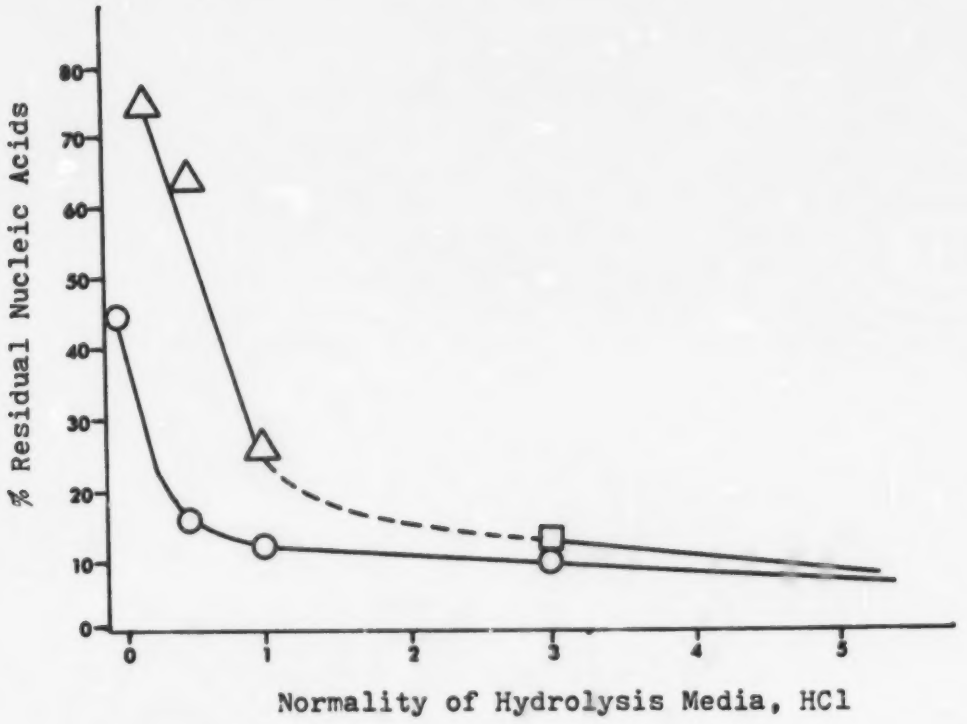


Figure 5. Effect of acid concentration upon hydrolysis of nucleic acids. Culture, Alcaligenes; hydrolysis temperature, 37°C., dialysis time, 12 hr., ○, Run #20, 12 hr. hydrolysis, □, Run #39, 12 hr. hydrolysis; △, Run #39, 10 hr. hydrolysis.

TABLE XIII

Composition of Single Cell Isolates

Run #	Hydrolysis[a] Media	Recovery Starting Material, %	Treated Isolate[b] Protein, %	Nucleic Acid, %
20[c]	H_2O[d]	79.5	75	8.9
20	H_2O	81	71	5.4
20	0.5N HCl	83.5	71	1.9
20	1.0N HCl	79.5	73	1.5
39[e]	H_2O	69	48	7.6
39	1.0N HCl	79	55	2.7
39[f]	1.9N HCl	82	--	5.9

[a] Hydrolyzed for 12 hours at 37°.

[b] After 12 hour dialysis with distilled water.

[c] Run #20, Alcaligenes grown on dextrose, homogenized, 78% protein, 9.55% nucleic acids.

[d] No hydrolysis

[e] Run #39, Alcaligenes grown on lactose, homogenized, 55% protein, 8.98% nucleic acids.

[f] Hydrolyzed for 1 hour at 50°C.

SECTION VIII

ULTRAFILTRATION AS A TECHNIQUE FOR REMOVING NUCLEIC ACID COMPONENTS

Apparently one of the areas for improvement in the previous purification scheme is the dialysis step. A means of providing a sharper molecular weight separation in a much faster procedure was desirable. The use of an ultrafiltration[16] step to replace the dialysis procedure was investigated (Table XIV).

TABLE XIV

Effectiveness of Ultrafiltration[1] in Removing Nucleic Acid Components After Acid Hydrolysis

Sample		% Sample Component in Retentate	% Sample Component in Filtrate
10% Casein, 1% Yeast RNA[2]	Casein	99	0.5
	RNA	83	17
10% Casein, 1% Yeast RNA[3]	Casein	99	1.0
	RNA	12	88
10% Cellulomonas[3]	Protein	95	5
	Nucleic Acids	16	83

[1]Amicon Model 52 Stirred Cell, PM 10 membrane (retains MW's greater than 10,000). Flow rate 0.5 ml/min, operating pressure 45 psi.

[2]Untreated sample.

[3]Hydrolyzed 12 hours at 37°C in 1N HCl.

An amicon magnetically stirred cell (Model 52, 50 ml capacity) with a one liter reservoir and selector valve was used. A PM10 membrane which retains molecular weights above 10,000 was selected for these experiments. A 50 ml sample containing 10% protein and 1% RNA was diluted to 500 ml after hydrolysis for 12 hours at 37°C in 1N HCl and this volume reconcentrated to 50 ml by ultrafiltration using a flow rate of 0.5 ml/min and an operating pressure of 45 psi. The nucleic acid analysis was done by UV absorbance[17] and protein determined by the Lowry Method.[10] The experiments were repeated using Cellulomonas. The results indicate that 95 percent of the protein can be retained while the nucleic acid

content of SCP reduced by over 80 percent. However, one of the greatest impediments to the use of ultrafiltration is concentration polarization, the accumulation of macrosolute at the membrane surface that both restricts flow and alters membrane selectivity. The type of apparatus used in these experiments operated at an optimum with a solute concentration of 1 percent, 10 percent being near the uppermost limit. These particular conditions would be impractical for use under industrial conditions.

SECTION IX

MOLECULAR WEIGHT DETERMINATION BY GEL CHROMATOGRAPHY

Since the ultimate objective of this project is to fabricate single cell protein, the physical properties of the protein component were examined. An effort was made to determine the average molecular weight and the molecular weight distribution (MWD) by gel filtration on Sephadex columns. The results were not very satisfactory; the protein mixture consists of a continous range of molecular weight components of equal concentration from approximately 2.5×10^5 to oligomers. Prefractionation of the samples by ultrafiltration before running them through the Sephadex columns reduced the width of the molecular weight distribution but the MWD was still too broad to determine the effect of the acid treatment on the MWD. An alternate and more definitive procedure was the use of purified protein samples of known molecular weight. The extent of degradation of two monodisperse enzymes, alcohol dehydrogenase and catalase was measured by gel permeation chromatography on Sephadex G-200.

The column, 2.0 x 90 cm, was packed by the method of Andrews.[18] A slurry of Sephades G-200 was allowed to swell for three days in a potassium dihydrogen phosphate-disodium hydrogen phosphate (pH = 7.8) buffer[14] before packing. A 0.02% concentration of sodium azide was present in the buffer as an antimicrobial agent. The slurry was degassed in a vacuum desiccator prior to packing. Column flow rate was maintained at 0.32 ml/min by means of a peristaltic pump. A 0.25% solution of Blue Dextran 2000, 3 ml, was used to test the homogeneity of the gel bed. The column was calibrated using 3 ml samples of the following proteins at a concentration of 5 mg/ml: Catalase (MW = 230,000), Alcohol Dehydrogenase (MW = 150,000), Bovine Serum Albumin (MW = 134,000) and Cytochrome C (MW = 12,400). The protein content of each fraction was analyzed by means of the Lowry Method.[10] Fractions of the column effluent were collected at 5 ml intervals. Figure 6 combines the gel chromatograms obtained for the untreated Catalase and Alcohol Dehydrogenase with those obtained after treating these two proteins with 1.0N HCl at 37°C for 12 hours (10% solution). This provides a means of assessing the effect of acid treatment on protein isolates from Cellulomonas and Alcaligenes. It was observed (Figure 6) that the molecular weight of the catalase drops from 230,000 to 10,300 after treatment with 1.0N HCl for 12 hours at 37°C. Under similar conditions the molecular weight of alcohol dehydrogenase is reduced from 150,000 to 45,000 indicating that relatively high molecular weight protein fragments remain intact in the acid media. The hydrolytic stability of the protein in dilute acid media is difficult to predict because the hydrolysis is not random. A high degree of specificity leading to preferential cleavage of aspartyl

residues is generally observed.[19] Since the protein isolate from single cell organisms is a complex mixture of enzymes with differing sensitivities to acid hydrolysis it would be expected that a significant broadening of the MWD would occur following the acid treatment. However, it is unlikely that this change in the physical properties of the protein mixture will reduce or effect its nutritional value. Further the presence of such an extensive concentration of low molecular weight protein in the isolate indicates that either the mixture must be fractionated before fabrication or the mechanical properties of the protein mixture must be supplemented by the addition of high molecular weight fillers during the fabrication process.

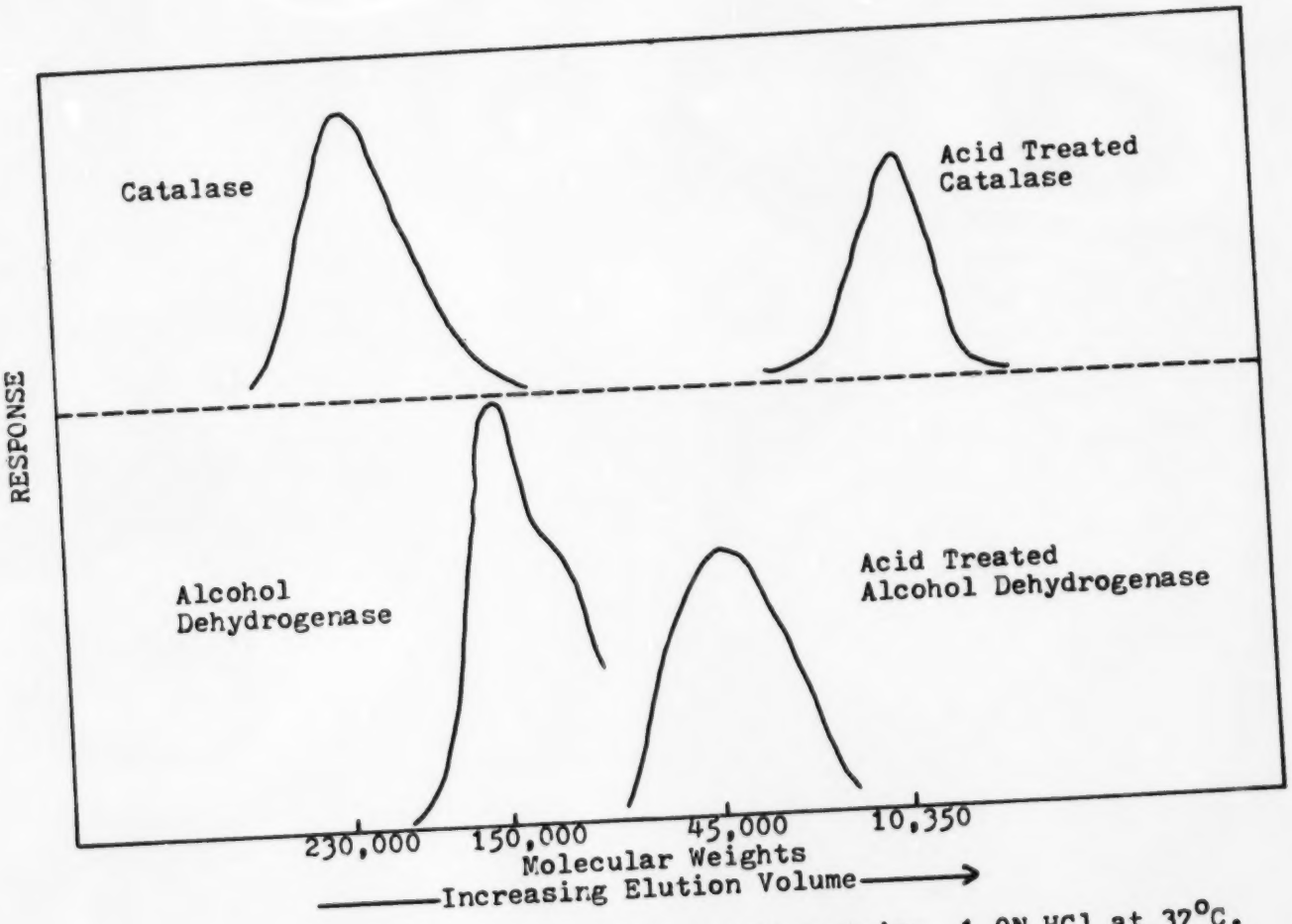


Figure 6. Gel chromatogram of acid treated proteins, 1.0N HCl at 37°C. for 12 hours.

SECTION X

THERMAL SHOCK TECHNIQUE FOR NUCLEIC ACID REMOVAL

Recent publications[20,21] on reduction of the nucleic acid content of yeast by a heat shock process prompted the investigation of the applicability of this procedure to bacteria. Since the presence of a ribonuclease is an apparent prerequisite for this process, the initial studies have been concerned with Cellulomonas only; work by Noriuchi[22] indicated that Alcaligenes faecalis lacks a thermally stable ribonuclease.

The technique used to test the influence of heat shock on nucleic acid degradation was developed by Maul et al.[20] and Ohta et al.[21] for use on yeast. Cell culture was pumped through stainless steel tubing (56 cm x 1/32" I.D.) by a peristaltic pump. This pump produced exact and reproducible hydrolysis times. The stainless steel tubing was immersed in a constant temperature bath controlled to within $\pm$ 0.1°C.

The bacterial cells and culture media were pumped through the tubing maintained at the appropriate shocking temperature and were collected in 15 ml centrifuge tubes. These tubes were cooled in an ice bath. Five to ten ml samples were used for each heat shock temperature. Immediately after collection the samples were placed in another constant temperature bath at the appropriate temperature for the incubation phase of the experiment. After incubation the samples were again cooled in an ice bath and centrifuged at 15,000 rpm and 0°C for 10 minutes. The supernatant was decanted, an aliquot (1 ml) of this was diluted to 10 ml with distilled water and the nucleic acid content determined by UV spectroscopy at 260 nm.[17] The residue was treated with 5 ml of 0.5N $HClO_4$ at 75° for 20 minutes, cooled in an ice bath and centrifuged as above. This extract was used to determine the amount of nucleic acids remaining in the cells also by UV spectroscopy. The optimum heat shocking temperature is shown in Figure 7, various heat shocking times and temperature are reported in Tables XV and XVI. Table XVII indicates the effect of culture age on the heat shock treatment. A reduction in the nucleic acid content of Cellulomonas through the use of the heat shock treatment based on the method of Maul et al.[20] is definitely possible, but the maximum reduction appears to be 50% of the initial content. As Table XV indicates, nearly 50% of the nucleic acids can be removed by heat shocking a viable cell culture at 72°C for 14 seconds followed by incubating the mixture for two hours at 52-53°C. The heat shocking temperature is more critical than the heating time. Varying the heat shocking temperature over a range from 65° to 80°C utilizing contact times of 5 to 15 seconds seemed to produce a significant increase in the effectiveness of the removal of the

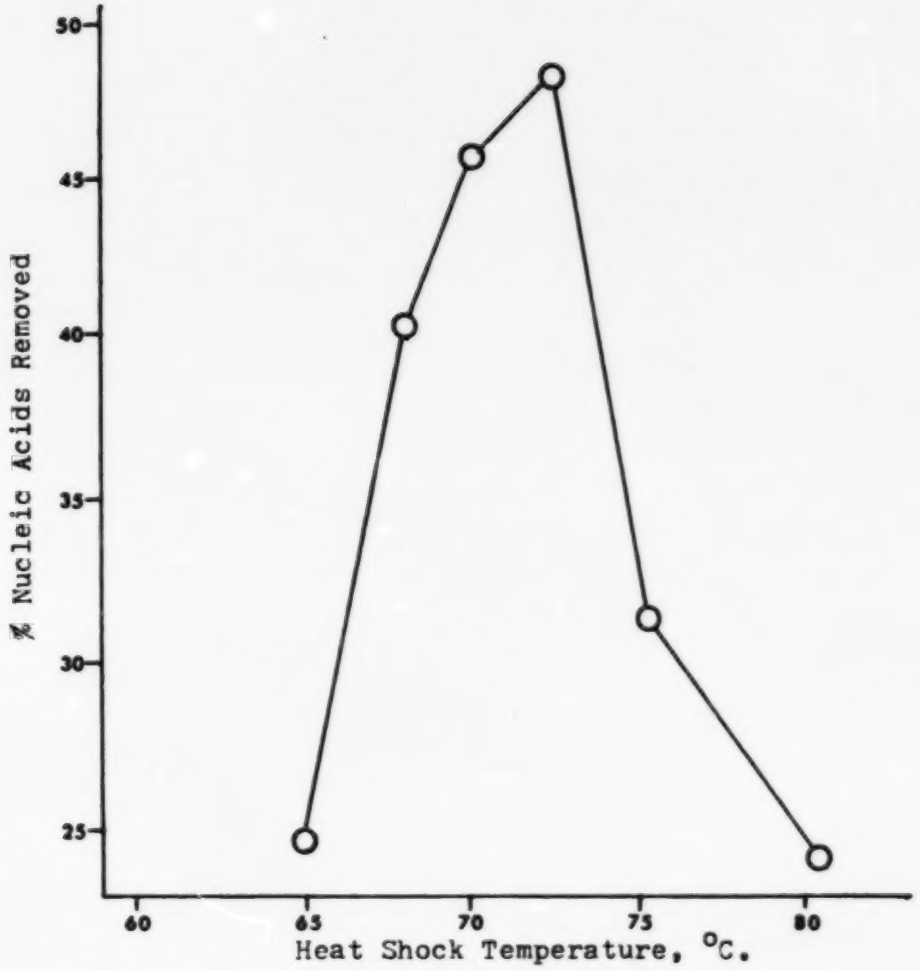


Figure 7. Effect of Heat Shock Temperature upon nucleic acid removal. Culture: Cellulomonas; age, 5 days; contact time, 10 seconds, incubation at 52-53°C. for 2 hours.

nucleic acids (Table XVI). Heat shocking at 68-74° from 5 to 18 seconds demonstrated that contact time of 14 to 15 seconds produced the optimum results but this is not a very sensitive variable (Table XV).

Heat shocking alone is not sufficient; an incubation period of at least two hours at 52-53° is required (Table XVI). The nucleic acids are hydrolyzed by the ribonucleases released by the heat treatment during this period. Centrifugation of the cell suspension immediately after heat shocking removes only 8-18% of the nucleic acid component; a similar amount can be removed by centrifugation of cell suspension after subjecting it to the incubation period only. Although the incubation period is necessary, variation in the incubation temperature from 46-61° failed to produce a significant change in the total amount of nucleotides removed. An incubation temperature of 56° was selected for further studies on the heat shocking process (Table XVIII).

TABLE XV

Reduction of Nucleic Acid Content at Various Heat Shock Times[a]

Heat Shock Temp.	Heat Shock Times			
	12 sec	14 sec	16 sec	18 sec
71°C	42.5%	42.8%	42.8%	43.6%
72	45.6	46.7	45.8	46.1
73	45.8	46.1	45.2	45.2
74	42.3	42.3	44.7	43.6

Heat Shock Temp.	Heat Shock Times		
	5 sec	10 sec	15 sec
68°C	34.3%	40.3%	40.3%
70	---	45.4	48.2
72	45.3	48.5	49.3

[a] *Cellulomonas*, 5 day culture; incubation, 52-53°C for 2 hours.

TABLE XVI

Reduction of Nucleic Acid Component by Heat Shocking and Incubation[a]

Heat Shock Time (sec)	Heat Shock Temp., °C	% Reduction in Nucleic Acid	% Reduction in Nucleic Acid by Heat Shock Only
-Incubate only-		14.5	
5	70	40.1	8.7
"	75	35.4	11.9
"	80	25.7	15.0
10	65°	24.7	3.9
"	70	41.9	10.1
"	75	31.3	11.9
"	80	24.1	17.8
10	68°	40.3	
"	70	45.4	
"	72.5	48.5	
15	68°	43.3	
"	70	48.2	
"	72.5	49.2	

[a] Cellulomonas; 5 day culture; incubation temperature: 52-53°C for 2 hrs.

TABLE XVII

Effect of Culture Age Upon Effectiveness of the Heat Shock Treatment[a]

Culture Age, Days	Heat Shock Time (sec)	Incubation Temp., °C	Reduction in Nucleic Acid, %
3	0	51°	5.1
	12	"	16.2
	15	"	15.9
	18	"	15.3
3	12	56°	19.8
	15	"	19.8
	18	"	18.2
	0	61°	15
	12	"	17.4
	15	"	16.5
	18	"	16.5
5	0	52°	14.5
	10	"	48.5
	15	"	49.2
9	0	56°	14.5
	12	"	14.5
	14	"	14.9
	16	"	14.9
13	0	56°	13.1
	12	"	15.5
	14	"	14.0
	16	"	14.0

[a] Cellulomonas; heat shock temperature, 72.5°C; incubation time, 2 hrs.

The most critical variable in this study proved to be the age of the cell cultures; the best results were obtained with five-day old cultures in the lag phase. As Table XVII indicates, three-day old cultures in log phase growth failed to respond to heat shocking. Older cultures, i.e. 9 and 13 days old, also failed to respond to the treatment. These results are consistent with those reported by Strange and Shon[23] in a study on Aerobacter Aerogenes. They observed the loss of RNA from their cultures after heating at 47° for one hour was dependent upon the composition of the liquid used to wash and resuspend the bacteria, the cell density, and the growth phase of the bacteria. Similar observations were reported by Allwood and Russell[24] when cultures of Staphylococcus Aureus were heated at 50 to 60° for one to two hours.

Further development of a heat shocking-incubation technique must await the establishment of the final conditions for continuous culture of a symbiotic Cellulomonas-B-glucosidase utilizing organism. This will fix the most critical variables, i.e. culture media, cell density, and the average age of the cells removed from the reactor. One would expect that the cells will be in log phase growth as they flow from the reactor so a holding period to assure that the cells enter the lag phase would be required if the heat shock treatment were to be employed. It may be possible to combine the heat shock technique with selective hydrolysis to achieve more efficient reduction in the nucleic acid content. A recent publication by Canepa et.al.,[25] which cites the symbiotic influence of high pH on the heat shock treatment of Saccharromyces cerevisae supports this hypothesis.

A further modification would be the use of homogenized cells for heat shocking. Ohta et.al.[21] mentioned this procedure and Sucher et.al.[26] has used the method successfully in the production of SCP concentrate from bakers yeast.

It is noted that in the section on acid hydrolysis (Figure 3) the use of EDTA resulted in increased loss of nucleic acid from the sample treated at 37°C for 12 hours. The addition of EDTA or other complexing agents may be another alternative to increasing the effectiveness of nucleic acid removal by heat shock.

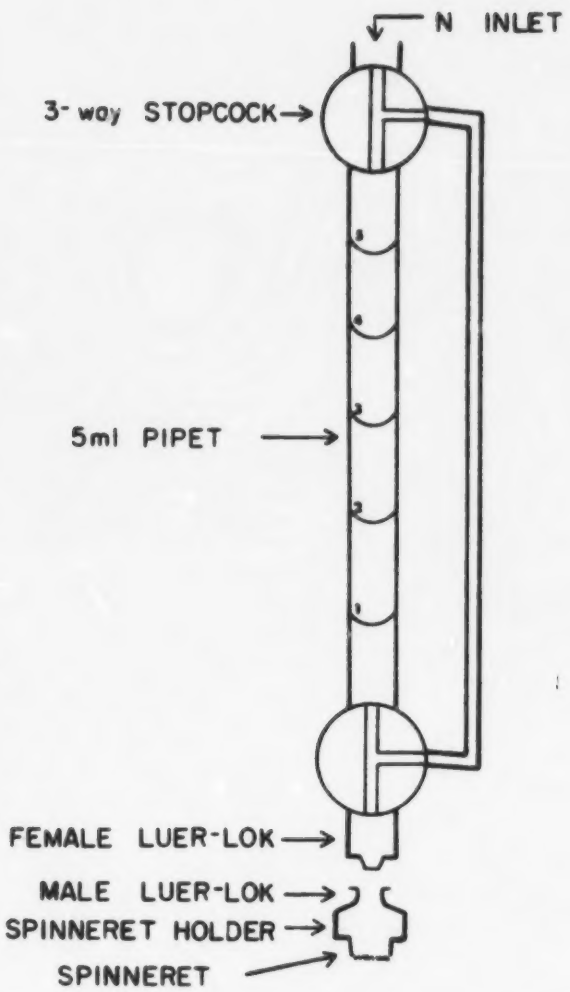
SECTION XI

RNA REDUCTION IN CONJUNCTION WITH PROTEIN FIBER FORMATION

The use of proteins for the production of synthetic fibers has been considered since before World War II,[27] but almost all of this research was concerned with the production of products for non-food use. It was not until 1954 that Boyer[28] texturized soy bean into a spun protein fiber that was intended for human consumption. Until very recently soy protein was the protein which was texturized most often for use in meat analog products.[29,30] In 1971, Huang and Rha,[31] studied the properties of a SCP concentrate with respect to dope formation and its flow behavior. Torula yeast was used to prepare a SCP dope containing 10 to 25 percent protein at pH 8, 9, 10, and 12. The study indicated that the concentrations of a SCP dope in the range of 15 to 20 percent was optimum for spinning and that maximum viscosity in the SCP dope was induced at around pH 9. Also in 1971, Heden *et al.*[32] spun bacterial proteins into fibers for the first time. The spinning dope was prepared by suspending disintegrated cells in a carbonate-bicarbonate buffer, pH = 9.5-11.0 for 2 hours at 5°C under continuous stirring. Fibers were spun from this solution both with and without removal of cell walls into a 0.7 percent propionic acid bath. A capillary tube spinning apparatus was used to obtain fibers of better quality than those obtained with a spinneret. It was found that the most suitable set of conditions for formation of fibers from bacterial protein were obtained when the arrangement was such that the protein solution was heat denatured at 80°C during its passage through a capillary tube. The strongest and most homogeneous fibers were obtained with pure desintegrated bacterial cells. Huang and Rha[33] in 1972 used alkali-extracted SCP concentrate from yeast to spin protein fibers. A capillary type spinning apparatus was also used. Spinning dope with a 20 percent SCP concentration at a pH of 9 gave the most desirable spinning characteristics.

The texturizing ability of *Cellulomonas* cells was tested using the spinning apparatus (Figure 8), a 5 ml buret modified to accept 0.5 inch spinnerets containing forty 0.005 inch orifices. The spinning dope was forced through the spinneret with positive nitrogen pressure (4 psi).

Cellulomonas cells grown on alkali treated bagasse were disrupted by passing a 10% cell suspension through a Manton-Gaulin Homogenizer. The SCP and cell debris were isolated by adjusting the pH of the suspension to 4 with 0.5N $HClO_4$, centrifuging and freeze-drying. The crude cell fragments, which contained 54% protein, 10%nucleic acids, 25% carbohydrates (cell walls and residual substrate) and



PROTEIN SPINNING APPARATUS

Figure 8

6% ash, was used to prepare the spinning dope. The disrupted Cellulomonas cells (15-20 wt %) were slurried in 0.5-1.0N sodium hydroxide solutions and incubated at 37°C for the times indicated in Table XVIII. Since the fibers formed from pure SCP were very weak, casein was added to the dope to improve the mechanical properties. Passage of the dope through the spinneret two to three times before extruding it into a coagulating bath removed air bubbles and gel particles and improved the flow characteristics. The dope (3 ml) was extruded into 50 ml of 0.5N $HClO_4$ at a rate of 4 cc/min, the fiber was drawn and allowed to soak in the acid bath for 5 minutes and then removed and freeze dried. The concentration of nucleic acids in the bath was determined spectroscopically (260 nm)[17] and the protein content of the fiber and the bath was determined by the Lowry method.[10] The efficiency of nucleic acid removal was confirmed using a casein dope containing 10% yeast RNA. The nature of the coagulating bath did not effect the results; nucleic acid removal was equally efficient in 0.5 HCl, citric acid-sodium citrate buffer, or $HClO_4$ at 0.25N to 1.0N.

The selective hydrolysis of nucleic acids with dilute alkali indicate (Table XVIII) that a 4-6 hour incubation time is sufficient to degrade the RNA components of the nucleic acids. The procedure does not require a high temperature alkali treatment; this minimizes the possibility of base catalyzed toxic by-product formation.[34] During the spinning operation the nucleotides dissolve in the coagulant bath, and the fiber acts as a microdialysis tube enabling nucleotides trapped within the fiber structure to difuse into the bath. Although it is possible to spin homogenized cell suspensions, very weak fibers are produced. The addition of casein as a high molecular weight carrier produced better fibers but very high casein concentrations (i.e. 16.5% of the total protein) were required. The addition of soybean protein or egg albumin did not result in fibers any stronger than those obtained by the addition of casein. Wheat gluten, CMHEC cellulose, and methyl cellulose did not appear to have any effect on strengthening fibers formed, nor did the spinning of the protein dope into a $CaCl_2$ solution. Solutions that contained lignin resulted in most of the lignin being trapped in the protein fibers. Samples that were hydrolyzed for as long as 20 hours showed little increase in RNA reduction. Also samples of spinning dope which were precipitated by acid and centrifuged lost approximately the same amount of RNA as the fiber spun samples. These results demonstrate that textured SCP with a nucleic acid content low enough to be utilized as a good food supplement can be produced by a suitably modified spinning process.

TABLE XVIII

Reduction of RNA in Cellulomonas by Alkaline Hydrolysis

Protein	Conc, Wt%	NaOH Conc.	Incubation Time,[a] hr.	Final Coagulation Bath Composition		Fiber Composition	
				% RNA[b]	% Protein[b]	% Protein	% RNA
Cellulomonas	18	1.0N	1	83	1.6	76	1.3
"	"	"	2	89	1.7	79	1.1
"	"	"	4	93	2.0	84	1.0
"	"	"	6	94	2.0	82	1.7
"	15	"	4	91			
"	20	0.5N	4	91			
"	20	"	6	96			
" + 4% casein	20	"	6	64			
" + 4% casein	20	1.0N	6	93			
Casein + 10% yeast RNA	10	0.3N	4	75			

[a] Slurry was agitated at 37°C.

[b] Wt % relative to initial sample composition.

SECTION XII

CELL FLOCCULATION

In Table XIX are the flocculating agents screened for possible use in a cell harvesting procedure. Stock solutions of all the polymers were prepared according to the manufacturers recommendations. The concentrations of the stock solutions were such that when approximately 1 ml of them was added to 100 ml of the culture media, the final concentration of the flocculant was in the desired range. Flocculant concentrations one order of magnitude on each side of the recommended dosages were also tested.

A modified jar test procedure by Wang and Gasner[35] was used to test the flocculants. A 1 ml sample of the flocculating agent was added to 100 ml of fresh culture media in a 250 ml flask. This mixture was shaken for 1 minute and the solution then poured into a 100 ml graduated cylinder. After 20 minutes, the reagents which had reduced the original cell volume by at least 50% were considered effective flocculating agents.

An actively growing culture of Cellulomonas grown on alkali treated bagasse with a cell density of 5.3 g/l dry weight was used. The culture media had a pH of 7.0 at the time of the test.

The results of screening 38 chemical flocculants as possible aids in harvesting Cellulomonas are in Table XIX. All of the flocculants tested failed to flocculate the cells in the culture media at the dosages recommended by the manufacturers (5-100 ppm range). Of the flocculants which were effective at a concentration of 1 g/l, the Betz Polymers were strongly cationic. The composition of the effective Nalco and Petrolite polymers was not available.

Earlier experiments[36] by the Nalco Chemical Company verify the fact that Cellulomonas cells in a fermentation media do not flocculate easily with polymers when the fermentation is at the harvesting stage. They reported that dissolved air flotation offered potential as a cell recovery technique.

Since the flocculation phenomena is an extremely complex one, further detailed study is needed to obtain the ideal flocculant or combination of flocculants which can be used to harvest the cells at a very low concentration. While the concentration of 1 g/l may first appear as a low value, in comparison with a cell concentration on the order of 10 g/l, the flocculant would be 10 percent of the harvested product. This could never be used on an industrial scale.

TABLE XIX

Possible Flocculating Agents for Cell Harvesting

Chemical Flocculants	Concentration[a] Range (ppm)
Betz Polymers, Betz Laboratories Inc.	50-0.5 [b]
Betz 1100, 1110, 1120, 1130, 1140, 1175 1190[c] and DK-522[c]	
Calgon Corporation	
Coagulant Acids 233, 243 and 253	100-1
Cat-Floc and Cat-Floc B	1000-10
General Mills Chemicals, Inc.	100-1
Gendriv 162	
Nalco Polymers, Nalco Chemical Co.	
Nalco 42-J Series 12, 13, 14, 15, 17, 18 and 21	50-0.5
Nalco 600, 605 and 610 107,[c] 603[c] and 607[c]	
Petrolite Polymers, Petrolite Corporation	1000-10
Tol-Floc 315	
Tol-Floc 300,[c] 305,[c] 310,[c] 320,[c] 325[c] and 330[c]	
Volclay Bentonite, American Colloid Company	500-5
Accofloc 350, 352 and 361	

[a] Based on manufacturers recommendations.

[b] Concentration per unit volume of culture media.

[c] Effective flocculants at a concentration of 1 g/l.

Another problem would be the inclusion in the SCP product of high concentrations of possibly toxic chemicals. Of all of the flocculants listed (Table XIX) only those produced by the Calgon Corporation are accepted by the Environmental Protection Agency for use in a product for human consumption. This is in potable water supplies at concentrations up to 1 ppm. Therefore, a chemical flocculant not only has to be effective at a low concentration, but should not remain in the SCP product at a concentration above 1 ppm.

There is also the possibility of using a natural ploymer such as casein as a flocculating agent. This would remove the possibility of possible inclusion of toxic chemicals in the SCP concentrate, since most chemical flocculants are not approved for food use. In Table XX are the results of flocculating cells with a 1.0% solution of casein.

The casein was added to the cell culture media to obtain a protein concentration of 1.0%. This solution was titrated with 1N HCl. The effect of pH on cell flocculation in this solution is in Table XX.

TABLE XX

Effect of 1% Casein on Cell Flocculation by pH Adjustment

pH	Effect on Cells[a]
7.0	No flocculation occurs
5.75	
5.50	Cells begin to agglomerate
5.25	Cells begin to settle from solution
5.00	Flocculated cells settle to 20 cc volume in 100 ml graduated cylinder

[a] Cellulomonas grown on alkali treated bagasse.

The experiment was repeated using a 0.5% casein concentration with similar results. If left standing for 12 hours the 1% casein solution at pH = 5.5 gave the same results as the solution at

pH = 5.0. Cells treated in a similar manner with no added protein remained completely in solution. The results at pH = 5.0 are comparable to using a chemical additive. Using a protein polymer would eliminate the need for removal of the flocculating agent during further processing. Also as all of the added protein is recovered in the product, this process should be fairly economical. By homogenizing some of the cells in the culture to release more free protein it may be possible to reduce the amount of added casein even further. The use of a protein with a higher isoelectric point would also result in a more economical process.

SECTION XIII

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The assistance and advice of Dr. V. R. Srinivasan and Dr. C.D. Callihan of the Louisiana State University Departments of Microbiology and Chemical Engineering respectively is acknowledged along with their contribution of supplying microbial cells when needed.

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SECTION XIV

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36. Unpublished Results

SECTION XV

PUBLICATIONS

W. H. Daly and L. P. Ruiz, Jr., "Selective Acid Hydrolysis of Nucleic Acids in Whole Cell Suspensions of Cellulose Utilizing Bacteria", Paper presented at the 162nd National Meeting of the American Chemical Society in Washington, D.C., Fall 1971.

W. H. Daly and L. P. Ruiz, Jr., "Reduction of RNA in Single Cell Protein in Conjunction with Fiber Formation", Accepted for publication in Biotechnology and Bioengineering.